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POLYMORPHISM OF CALPASTATIN, CALPAIN AND MYOSTATIN GENES IN NATIVE DALAGH SHEEP IN IRAN

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ABSTRACT

Calpains play a major role in post mortem tenderization and calpastatin is the endogenous inhibitor of calpain proteases and regulates the rate and extent of post mortem tenderization. Myostatin is an inhibitor of skeletal muscle growth and a mutation in the gene coding region leads to increased muscling. Therefore, they are considered as candidate genes for meat and growth traits. Blood samples were collected from 110 Dalagh sheep and DNA were extracted using modified salting out extraction method. Genotypes were determined by *PCR* amplification followed by single-strand conformation polymorphism (*SSCP*) method for calpain gene and restriction fragment length polymorphism (*RFLP*) method for calpastatin and myostatin genes. Based on results, calpastatin and calpain genes were found to be polymorphic but myostatin gene was monomorphic. Genotype frequencies were 36, 38, and 26 % for *MM*, *MN* and *NN* in calpastatin locus, respectively. In this population, calpastatin locus did not show Hardy-Weinberg equilibrium ($P<0.05$). Observed heterozygosity for this locus was good (0.38). Under the *SSCP* analysis, three different patterns (G_1, G_2 and G_3) with frequencies of 8.2, 89.1, and 2.7 % were detected in calpain locus, respectively. Detected polymorphisms and assumed associations of genetic variation with meat production and tenderness may help to find the effective genotypes of Dalagh sheep for those economic traits.

Key words: calpastatin; calpain; myostatin; molecular methods; polymorphism; sheep

INTRODUCTION

Considerable progress in farm animal breeding has been made in the last few decades, but achieving greater understanding in the improvement of meat quality was very slow before molecular markers became an accessible technology with wide applications in breeding methods (Gao *et al.*, 2007). In case of sheep, research on genetic polymorphism of three candidate genes, calpastatin, calpain (Palmer *et al.*, 1999) and myostatin have been carried out (Kambadur *et al.*, 1997). The calpain-calpastatin system (*CCS*) contains a family of Ca^{2+}-dependent neutral proteases. This system is found in most animal tissues and influences many important processes including muscle development and degradation, meat tenderization post mortem, cataract formation and fertility (Palmer *et al.*, 1999). Calpastatin and calpain deserves special attention because of their major role in meat production and quality. Calpastatin (*CAST*) is the endogenous and specific inhibitor of calpain proteases and regulates the rate and extent of post mortem tenderization (Kocwin and Kuryl, 2003). Calpain (*CAPN*) plays a major role in post mortem tenderization in beef, lamb and pork by degrading structure of muscles (Huff-Lonergan *et al.*, 1996). A number of studies have shown that the calpain system is also important in normal skeletal muscle growth (Palmer *et al.*, 1997; 1999; Goll *et al.*, 2003). An increased growth rate of skeletal muscle may result from a decreased rate of muscle protein degradation due to reduction of calpain and increase in calpastatin activities. Myostatin (*MSTN*) or growth differentiation factor-8 (*GDF-8*) is a member of the mammalian growth transforming family (*TGF-beta superfamily*), which plays a role in the regulation

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of embryonic development and tissue homeostasis in adults (Sonstegard *et al*., 1998). They are known to block myogenesis, hematogenesis and enhance chondrogenesis as well as epithelial cell differentiation *in vitro*. In mice, null mutants are significantly larger than wild-type animals, with 200–300 % more skeletal-muscle mass, because of hyperplasia and hypertrophy (McPherron *et al*., 1997). Muscular hypertrophy (*mh*), also known as "double-muscling" in cattle, has been recognized as a physiological character for years (Arthur, 1995) and is seen in Belgian Blue, and Piedmontese cattle (Kambadur *et al*., 1997). These animals had less bone, less fat, and 20 % more muscle on an average (Shahin and Berg, 1985; Hanset, 1991; Casas *et al*., 1998). Mutations within myostatin gene led to muscular hypertrophy allele (*mh allele*) in the double muscle breeds (Kambadur *et al*., 1997). Such a major effect of a single gene on processing yields opened a potential channel for improving processing yields of animals using knockout technology (Arif *et al*., 2002). Therefore, sequencing of calpastatin, calpain and myostatin genes of farm animals is important to produce genomic resources for development of knockout technology as well as for understanding the structure, function and evolution of the gene.

In Iran, sheep meat is a major source of animal protein and investigation for meat quality and related genes is important. Dalagh is a kind of fat-tail sheep and also known as Semi fat-tailed Turkmen or Atabai. This breed is found mainly in the northeast region of the Turkmen plain, located in Golestan Province. The sheep is resistant to humid environment and parasites (Saadatnoori and Siahmansoor, 1990). The aim of present study was to identify genotypes of calpastatin, calpain and myostatin genes in Dalagh sheep using *PCR-RFLP* and *PCR-SSCP* methods in order to find effective alleles influencing meat quantity and quality traits in sheep.

MATERIAL AND METHODS

Animals and DNA extraction

Blood samples were randomly collected from 110 Dalagh sheep from Golestan province. DNA was extracted from blood as described by Miller *et al.* (1988). Quality and quantity of DNA were measured by visual and spectrophotometer methods.

PCR

Two pairs of primers were used for amplifying each of *CAST*, *CAPN* and *MSTN* loci using primers suggested by Nassiry *et al.* (2007) and Timothy *et al.* (1997), respectively. The primer sequences are presented in Table 1. An aliquot of 100 ng genomic DNA was amplified in a total volume of 15 µl *PCR* mix. The *PCR* mix consisted of: 7.5 µl Master mix (Cinna clon, Iran), 2 µl forward and reverse primers (10 pmol/ µl), and 4.5 µl ddH_2O. Amplification conditions are shown in Table 2.

In every experiment, negative controls were used, aiming to avoid contaminations. Assays were performed in a thermal cycler (Personal Cycler™ - Biometra, CA, German), and the amplicons were analyzed by 1.5 % agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized under ultraviolet light.

Digestion reaction

10 µl of *PCR* products were incubated for 10 h at 37 ^{o}C with 1 µl (10 units) of *MspI* and *HaeIII* enzymes for calpastatin and myostatin genes, respectively. Digestion products were separated by electrophoresis on 2 % agarose gel, stained with ethidium bromide for calpastatin and 8 % non-denaturing polyacrylamide gels, stained by silver nitrate staining method for myostatin genes, respectively (Benbouza *et al*., 2006) (Figures 1 and 3).

Table 1: Locus, region, methods, primer sequence (5'→3') and length of *PCR* products of the *CAST*, *CAPN* and *MSTN* genes

Locus	Region	Method	Primer sequence (5'→3')	Length of fragment (bp)
CAST	Exon and intron1	*PCR-RFLP*	F: TGGGGCCCAATGACGCCATCGATG	622
			R: GGTGGAGCAGCACTTCTGATCACC	
CAPN	Exon 5 and 6 including intron	*PCR-SSCP*	F: AACATTCTCAACAAAGTGGTG	190
			R: ACATCCATTACAGCCACCAT	
MSTN	Exon 3	*PCR-RFLP*	F: CCG GAG AGA CTT TGG GCT TGA	337
			R: TCA TGA GCA CCC ACA GCG GTC	

F: forward and R: reverse

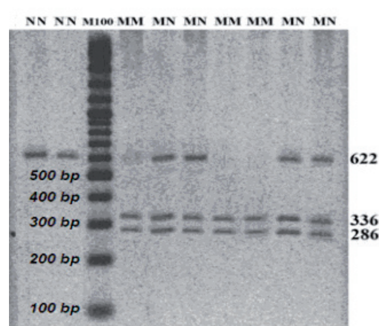

Fig. 1: Restriction patterns of 622bp fragments of *CAST* gene after digesting with *MspI* on 2 % agarose gel and staining with ethidium bromide. Molecular marker was *M100*.

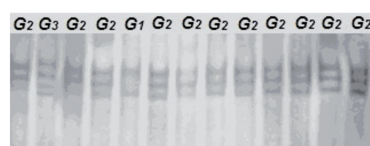

Fig. 2: The *SSCP* patterns of 190 bp fragments of the ovine *CAPN* regulatory gene, on 8 % non-denatured polyacrylamide gel after silver nitrate staining. Three patterns demonstrating the 3 genotypes are presented.

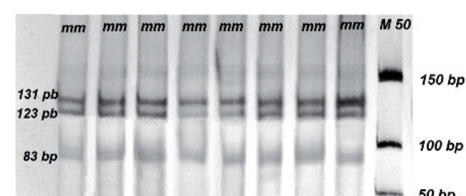

Fig. 3: Restriction patterns of 337 bp fragments of *MSTN* gene after digesting with *Hae*III on 8% non-denatured polyacrylamide gel after silver nitrate staining. Molecular marker was *M50*.

Table 2: *PCR* conditions

Locus	Primary denaturation in 1st cycle	Denaturation		Annealing		Elongation		Final extension	Number of cycles
	°C/Sec	°C	Sec	°C	Sec	°C	Sec	°C/Sec	N
CAST	95/180	95	60	59	60	72	120	72/420	35
CAPN	95/180	94	45	59	60	72	75	72/600	35
MSTN	94/240	94	60	58.5	60	72	120	72/240	35

Table 3: Allele and genotype frequencies; observed, expected and average heterozigosity for *CAST* and *CAPN* loci

Locus	Allelic frequencies[a] (%)		Genotype frequencies[b] (%)			Heterozigosity			χ^2
	A_1	A_2	G_1	G_2	G_3	Obs.	Exp.	Ave.	
CAST	55.45	44.55	36	38	26	0.38	0.49	0.49	5.908*
CAPN	-	-	8.2	89.1	2.7	-	-	-	-

a: A_1 and A_2 correspond to M and N alleles for CAST locus

b: G_1, G_2 and G_3 correspond to MM, MN and NN genotypes for CAST locus. *: P<0.05

SSCP

Genotyping of calpain locus was performed by *PCR-SSCP* method. *PCR* products (3 µl) were diluted with 13 µl of running buffer (including 800 µl formamide 99 %, 100 µl loading dye, 100 µl glycerol 98 %, 3 µl 0.5M *EDTA* and 2 µl 10M NaOH). After heating at 95°C for 5 min, they were immediately placed on ice for 10 min. Polymorphisms were detected using 10 % non-denaturing polyacrylamide gels (Figure 2). The mixture was electrophoresed for 4 h at 250 V and 10°C. DNA fragments were visualized using the silver nitrate staining method (Benbouza *et al.*, 2006).

Calculation of genotypes and allele frequencies, expected and observed heterozygosity and examination of Hardy-Weinberg equilibrium were performed using PopGene32 (Ver. 1.32) (Yeh *et al.*, 2000) (Table 3).

RESULTS AND DISCUSSION

CAST

A 622 bp fragment from *CAST* was amplified. The *MspI* restriction enzyme digested the *PCR* products and alleles of *M* and *N* were detected. The *MspI* digests the allele M, but not allele *N*. The *MspI* digestion of the allele *M* produced digestion fragments of 336 and 286 bp (Figure 1). The allelic frequencies were 55.45 and 44.55 % for *M* and *N*, respectively. The genotype frequencies in Dalagh sheep were 36, 38, and 26 % for *MM*, *MN* and *NN*, respectively (Table 3). In this population, this locus didn't show Hardy-Weinberg equilibrium ($P<0.05$) (Table 3). Observed heterozygosity for this locus was good (0.38) in the herd (Table 3).

CAPN

The ovine calpain regulatory gene, exon 5 and 6 including intron (*CAPN*456), with 190 bp length was amplified. Under the *SSCP* analysis, different conformations were detected by electrophoresis on non-denaturing polyacrylamide gel (Figure 2). Genotype frequencies were 8.2, 89.1, and 2.7 % for G_1, G_2 and G_3, respectively (Table 3).

MSTN

A 337 bp fragment for exon 3 of *MSTN* locus was amplified. *HaeIII* restriction enzyme was used to digest the *PCR* products. The *HaeIII* digests the m allele, but not *M* allele. Digestion of the m allele produced three fragments of 83, 123, and 131 bp (Figure 3). All samples were digested by *HaeIII* enzyme and showed the mm genotype. As a result, all of them were monomorphs (Figure 3).

Results showed polymorphism in *CAST* and *CAPN* loci but *MSTN* locus was monomorphic. Three different genotypes (*MM*, *MN* and *NN*) were showed in *CAST* locus. Similar result for calpastatin locus was observed in Iranian Karakul sheep by Eftekhari Shahroudi *et al.* (2006). A high degree of calpastatin polymorphism has also been reported in studies on Dorset Down hoggets, Dorset Down×Coopworth sheep, Corriedale rams and Angus bulls (Palmer *et al*., 1997; Chung *et al*., 1999). Palmer *et al.* (1997) detected three genotypes (*MM*, *MN* and *NN*) in unrelated Corriedale rams for this locus which was in agreement with the present results. Chung *et al.* (1999) observed *AA*, *AB* and *BB* genotypes for *CASTI* and *CAST5* loci, and *AA*, *BB*, *CC*, *AB*, *AC* and *BC* genotypes for *CAST10* locus in Angus bulls. In this population, this locus didn't show Hardy-Weinberg equilibrium. This confirmed that factors leading to disequilibrium, especially selection, may affect into genetic structure of the population. Based on our results, the investigated population showed a good degree of genotypic variability for the *CAST* gene. This may be explained by the conservation and breeding strategies, which have been carried out.

The calpain gene was investigated as a potential candidate gene for quantitative trait locus (*QTL*) affecting meat tenderness (Chung *et al*., 1999). Under the *SSCP* analysis, three different patterns (G_1, G_2 and G_3) were separated by electrophoresis on non-denaturing polyacrylamide gel. Genotype frequencies for G_1, G_2 and G_3 genotypes were 8.2, 89.2, and 2.7 %, respectively. These results were similar to those obtained by Tahmoorespour *et al.* (2006), where they found three genotypes (*AA*, *AB*, and *BB*) in Baluchi sheep. In contrast to our result Nassiry *et al*. (2007) found two genotypes (*AA*, and *AB*) in Kurdi sheep.

In myostatin locus, all samples were digested by *HaeIII* enzyme and showed the mm genotype. As a result, all of them were monomorphic. On the contrary Soufy *et al.* (2009 a and b) observed polymorphism for *MSTN* gene in Sanjabi Sheep and native kermanian cattle. This inconsistency may be ascribed to breed differences, population and sampling size, environmental factors, mating strategies, geographical position effect and frequency distribution of genetic variants.

Although myostatin locus was monomorphic in the herd, but results showed acceptable polymorphism for calpastatin and calpain loci, which may open interesting prospects for future selection programmes, especially using marker-assisted selection for improving weight gain and meat quality.

Furthermore, results showed that *PCR-RFLP* and *PCR-SSCP* techniques are appropriate tools for screening *CAST* and *CAPN* loci in sheep breeds. This was one of the first studies on polymorphism of calpastatin and calpain loci in Dalagh sheep. Due to lack of suitable phenotypic records it was not possible to observe association between different genotypes and animal production. Hence, detected polymorphisms and assumed associations of genetic variation with meat production and tenderness may help to find the effective genotypes of Dalagh sheep for such economic traits.

CONCLUSION

The goal of this study was to determine genetic polymorphism of calpastatin (*CAST*), calpain (*CAPN*) and myostatin (*MSTN*) genes in Dalagh sheep. These results open up interesting prospects for future selection programmes, especially marker assisted selection. Results also confirmed that *PCR-RFLP* and *PCR-SSCP* are appropriate tools for evaluating genetic variability.

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